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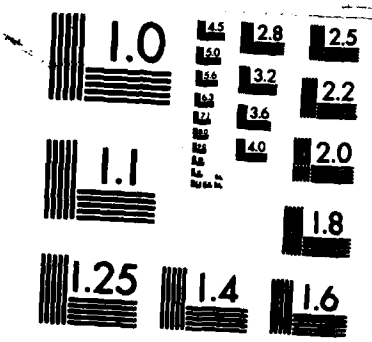

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

N-acetylgalactosaminidase, an enzyme which can convert type A human red blood cells to type O has been purified from human placenta by ammonium sulfate fractionation, isoelectric precipitation of impurities and affinity chromatography. A similar enzyme from the culture medium of Clostridium paraputrificum has been prepared by ammonium sulfate fractionation, gel filtration and affinity chromatography. Its effect on the conversion of type A cells and its properties have been investigated.

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PURIFICATION OF N-ACETYLGALACTOSAMINIDASE

BY ISOELECTRIC FOCUSING

Contract No. N00014-83-K-0339

Work Unit No. NR 464-005

Annual Report

April 15, 1984-April 14, 1985

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Principal Investigator

Department of Biochemistry

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This contract is directed at the enzymatic conversion of type A red blood cells to type O cells which can be used in all recipients. This would simplify the logistics of blood storage and utilization. Blood group A substance has an alpha linked N-α-acetylgalactosamine at its non-reducing terminus. Removal of this residue yields O antigen. The enzymes which perform this function, alpha-N-acetylgalactosaminidases, are referred to as Azymes in this report. Progress during this period has been concerned especially with perfecting the affinity purification of Azymes and in converting type A cells to type O. Studies with Azyme from Clostridium paraputrificum have shown that small amounts of enzyme remove much of the A activity and that the partially purified enzyme has very little sialidase activity.

I. Conversion of Type A Red Cells to Type O

The Azyme from Clostidium paraputrificum removes A activity from type A red cells (Fig. 1). The rate of loss is related to enzyme concentration. One of the unexpected findings was that the removal leveled off after a period of time. This was not due to destruction of the enzyme. After 48 hrs at 37° followed by 2.5 days at room temperature most of the original activity remained. We then considered that erythrocyte metabolism (ie. the formation of lactic acid from glucose) might acidify the reaction beyond its optimal pH. This too was found not to be the case. The initial pH used was 6.80 and the final pH after the time above was 6.79±0.02 in 9 determinations. We then considered that the end product might be inhibiting the reaction. N-acetylgalactosamine proved to be a good inhibitor but is probably not the cause of the leveling off of the reaction. Calculation of the amount expected to be liberated in relation to the K_I suggestes that this is not the problem. Failure to increase the rate of reaction by replacing the enzyme solution with fresh solution further suggests that this is not the cause.

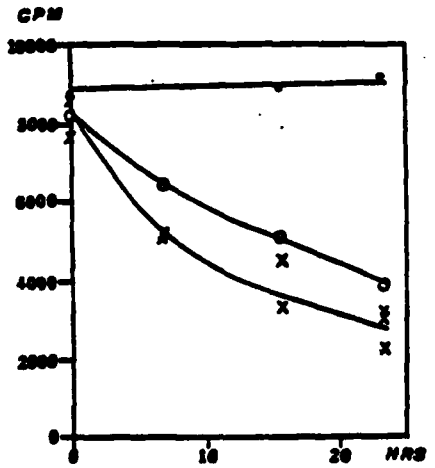


Fig. 1. Effect of Clostridial Enzyme on Type A Red Cells
The reaction mixture contained 125 ul of packed red cells, 100 ul of gel filtered clodtridial Azyme containing 0.0073 units (O) (uM/min) or twice this many units (X), isotonic phosphate (25 mM) buffered saline pH 6.8 and 50 ul of isotonic CaCl2 (7 mM). The cells from 1 ul of reaction mixture were used for each determination. Solid circles have buffer in place of enzyme. Controls with O cells (not shown) averaged 210 cpm.

Three minor problems were encountered in getting the conversion to occur. All involved hemolysis. The reaction mixture described by Paoni recommends that 1/6 of the reaction mixture be 7mM $CaCl_2$. Addition of sufficient NaCl to bring its osmolarity to 300 mOsm greatly reduced hemolysis. In experiments with crude ammonium sulfate fractions, the Azyme itself was slightly hemolytic. This tendency which was mild disappeared after gel filtration of the enzyme. It is not clear whether the problem was associated with a hemolysin or with substances in the medium. At the suggestion of a colleague, the stability of the cells was further improved by the addition of glucose to the reactions. The final concentration of glucose was 37.5 mM.

II. Affinity purification of Azymes

The affinity matrix previously used deteriorated after several months of storage at $4^{o}C$ in the presence of 0.02% NaN_3 and it was necessary to synthesize a new batch. This proceeded uneventfully. Preliminary tests showed that the new material survived freeze drying and the bulk of the new material has been stored in this form at -20^{o} over silica gel. It seems probable that the affinity matrix lost activity because of hydrolysis of the substituted amide linking the sugar derivative with the "arm" of the matrix. Preliminary tests have been done with Azyme from a DEAE fraction from human placenta, from the gel filtration step of a clostridial preparation and from partially purified material from Charonia lampas. All were absorbed. The placental enzyme was absorbed from citrate phosphate buffer pH 4.7 containing 26.7mM citric acid, 46.6mM Na_2PO_4 and 150mM NaCl. For the clostridial enzyme the buffer was 0.01 M phosphate pH 7.0 containing 0.01mM DTT. The Charonia lampas enzyme was absorbed from pH 4.0 citrate-phosphate containing 30.7mM citric acid, 38.6mM Na_2PO_4 and 500mM NaCl. These conditions were chosen because they are those used for assay and accordingly those where one would expect a high interaction with substrate.

Several experiments have been done with the new batch of affinity matrix to establish its use at an earlier stage in the purification of placental Azyme. Initial experiments with a 0.5x5 cm (1ml) column give consistently satisfactory results (Fig. 2). A 2.5 ml sample of DEAE purified material which had been freeze dried was dissolved in a citrate phosphate buffer (citric acid 21.81g, disodium phosphate 28.45g /4 liters, pH 4.7). A suspension of dried enzyme was dialyzed overnight, centrifuged and applied in a volume of 2.5 ml at a flow rate of 3 ml/hr. The column was washed with 70 ml of the buffer prior to sample application and eluted with a 7 ml linear gradient going from 0 to 0.5 M NaCl in the buffer after a 7 ml wash with the initial buffer. In the experiment shown, 44% (0.15 U) of the applied activity was recovered in the main peak. The purification was 346 fold. Attempts are being made to scale up the preparation. We are anxious to do the affinity step at this point in the scheme to avoid the tedious chromatographic separation on hydroxylapatite.

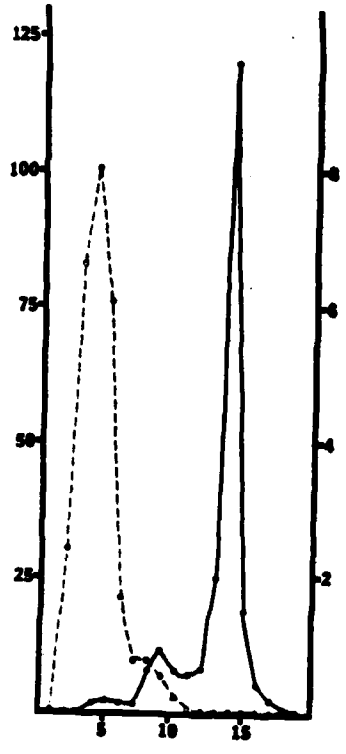


Fig. 2 Affinity Purification of DEAE Purified Placental Enzyme
Azyme activity (———) and protein (-----) are plotted against fraction number. Fraction volume 1.2 ml. Initial load 0.34 U. Other conditions as described in the text.

The clostridium enzyme was absorbed at pH 6.2 in citrate-phosphate buffer. The affinity column was washed with 41 ml of the citrate-phosphate buffer and the enzyme was eluted with a 40 ml linear salt gradient going to 0.5M NaCl in pH 7 citrate-phosphate buffer preceded by a 20 ml step gradient to 0.4 M galactose in the buffer. The affinity column was finally washed with 20 ml of 0.4M N-acetylgalactosamine. A small amount of additional Azyme was removed with the last eluant (Fig. 3). For this experiment a 1.0x 10 cm column was used. In the experiment shown, 53% of the applied activity was recovered in the peak tube with a purification of 33 fold.

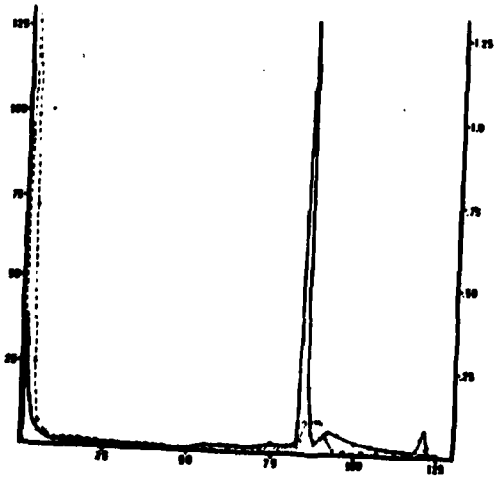


Fig. 3 Affinity Purification of the Clostridial Azyme from the Gel Filtration Step
Protein (-----) and Azyme (———) are plotted against the volume of eluate. Other conditions are described in the text.

III. Studies with Azyme from Clostridium paraputrificum

The blood group degrading activity of the clostridial enzyme has been previously examined by Nicholas Paoni and his coworkers at the Naval Biosciences Laboratory in Oakland, CA. Cultures and samples of partially purified enzyme were obtained from Joan Quay at NBL. Dr. Bong Park, a Visiting Scientist, has undertaken the culture of the organism and studied the early steps in purification of the enzyme. Dr. Park is a volunteer and not supported by the contract. He has optimized the time and temperature for growth and has found that times longer than the 13 hr suggested by Paoni et al are better that shorter times. At high temperatures the organism sporulates and the spores are also a satisfactory innoculum. The organism is not harmed by less than rigorous anerobiasis. Cultures plugged with cotton or covered with loose fitting caps grow and produce well. He has shown that the bacterium grows and produces Azyme in a synthetic medium. While fractionation with ammonium sulfate has been studied, for most experiments the enzyme has been precipitated at 90% saturation to avoid any loss of enzyme in case more than a single activity occurs. Gel filtration on Sephacryl S-300 has been used next (Fig. 4).

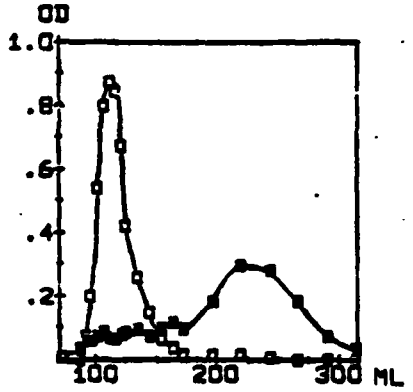


Fig. 4 Gel Filtration of Clostridial Enzyme
Fractions (4.7 ml) were collected from a 2.5x80 cm column of Sephacryl S-300 loaded with a five ml sample of ammonium sulfate precipitated enzyme and analyzed for Azyme (open squares) and protein (solid squares).

The effect of the concentration of medium on Azyme production was examined. It was found that Azyme production is about twice as great at 2/3 the concentration of medium. This material also filters much more readily through the 0.45 u Millipore filter used to prepare the culture supernatant. The specific activity of the Azyme from a 10 day culture was independent of the concentration of the medium although concentrations less than 1/2 of the original gave decreased amounts of enzyme.

Perhaps the most promising aspect of the work with the clostridia is the studies done with media developed for Neisseria gonorhoeae by Morse and Bartenstein (Can. J. Microbiol. 26 13-20 (1980)) was used. It is a mixture of salts including $CaCl_2$ and $Fe(NO_3)_3$, amino acids, thiamine, pantothenate, biotin, glucose, hypoxanthine and uracil. For Azyme production it is necessary to add 0.02% pig gstric mucin, the only undefined constituent. A study optimizing culture duration is underway.

The sialidase content of the Azyme from Clostridium paraputrificum has been examined. The partially purified enzyme

appears to be sialidase free. This supports the findings of Paoni et al. The purity of the preparations and difficulties with the assay are important factors. The thiobarbituric acid method was used. Aminoff and Paoni also used this method. Paoni found no detectable sialidase in the partially purified material. Neither do we. Four spectra from the assay indicate one of the problems with the assay (Fig 5). In A the spectrum of the product of Clostridium perfringens sialidase action on fetuin (- - - -) is compared with a sialic acid standard (———) and a blank (· · · · ·). The spectra are quite similar. In B the products formed by the action of a filtrate from a Clostridium paraputrificum culture (- - - - -) are compared with the sialic acid spectrum (———) and a blank which contained no fetuin (· · · · · · ·). The filtrate contains some colored substance which is clearly not sialic acid. It is difficult to rule out a small amount of sialic acid. The worst case estimate, assuming that all of the optical density formed at 549nm is due to sialic acid release, would be .088 units of sialidase per unit of Azyme. Frame C shows comparable data for material recovered by gel filtration. There is no discernable sialidase. We believe that there are less than 4×10^{-5} units per unit of Azyme. Frame D gives data with affinity purified enzyme. While there is a small difference between control (· · · · ·) and Azyme (- - - - -) the lack of any peak at 549nm suggests that again little sialic acid is present. Less Azyme was used in the experiment so that the top limit is .0023 units per unit of Azyme. After ammonium sulfate purification, gel filtration and DEAE purification, Aminoff had .77 units per unit of Azyme from Clostridium perfringens.

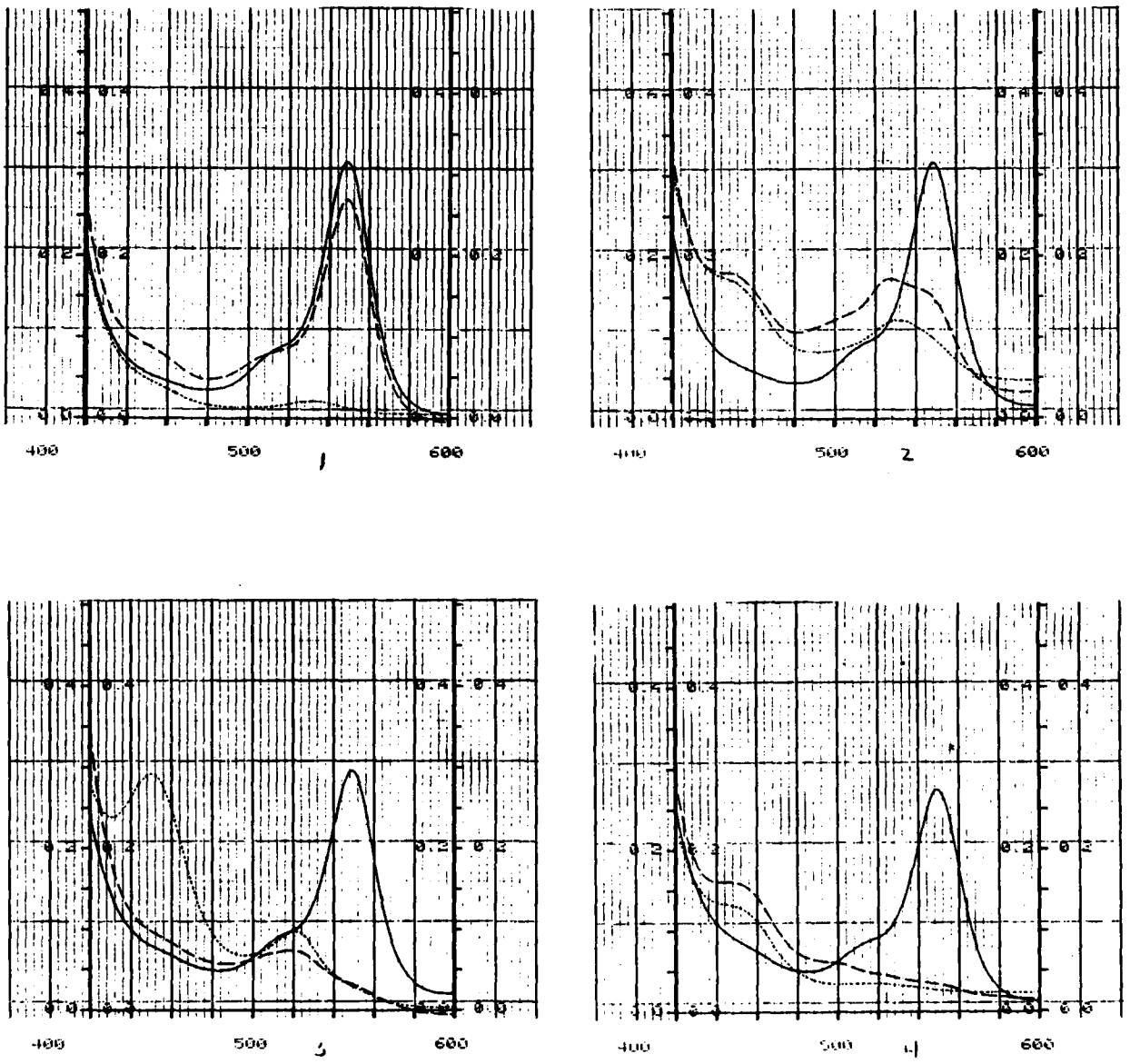
Properties of Azyme from Clostridium paraputrificum

A number of the kinetic and enzymatic properties of this Azyme were examined by Sunita Sarin a senior at Kalamazoo College as part of a senior project. She was not supported by ONR funds. Portions of the work were done to confirm the findings of Paoni et al and to assure that the same enzyme is being examined. All were done with the synthetic substrate p nitrophenyl-2-acetamido-2-deoxy-α-D-galactopyranoside (Kock-Light).

pH optimum

The pH optimum of the partially purified enzyme prepared by ammonium sulfate precipitation and gel filtration was determined at 37° using a series of 0.01M buffers between pH 4 and 9. Sodium acetate buffer was used at pHs acid to 5.8 and Tris acetate was used above pH 7.8. Sodium phosphate buffers were used for the other pHs. As indicated in Fig. 6, the optimal pH was between pH 6.0 and 6.5.

Fig. 5 Sialidase Assays on Clostridial Azyme



The absorbance is plotted against the wavelength. The thiobarbiturate assay was used with sialidase from Clostridium perfringens (A), with culture filtrate from Clostridium paraputrificum (B), with the latter enzyme purified by gel filtration (C) and affinity chromatography (D). The solid lines are sialic acid standards, the dashed lines are the assays and the dotted lines are controls which contained no substrate.

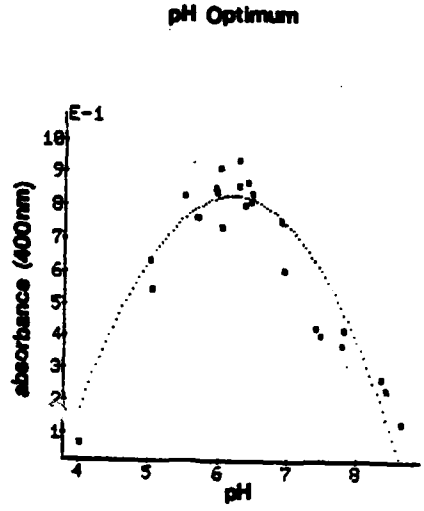


Fig. 6 pH Optimum for Clostridial Azyme
The activity is plotted aginst the pH. A portion of the scatter is due to the fact that a number of experiments done at different times have been plotted. Other details are given in the text.

Kinetic parameters at various temperatures

The velocity was determined at a series of temperatures using substrate concentrations of 0.64, 0.91, 1.60 and 3.20 mM at pH 7 in 0.01M sodium phosphate buffer containing 0.1 mM dithiothreitol and 0.68 mM $CaCl_2$.

Temperature	Km	Vmax
17	.19	6.69×10^{-8}
23	.30	1.46×10^{-7}
30	.62	2.55×10^{-7}
37	.88	2.73×10^{-7}
44	1.14	5.66×10^{-7}

An Arrhenius plot of this data gave an energy of activation of 12,580 cal/mole.

Calcium requirement

Enzyme assays under the above conditions were done at 3.20 mM substrate at calcium levels between 0 and 3.4 mM. Samples incubated overnight in 1 mM EDTA and ran in its presence had the same activity as those done in the absence of Ca^{++}. The activity was increased about 50% by the calcium (Fig.7).

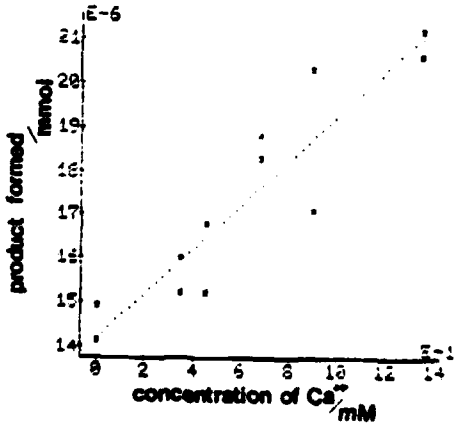


Fig. 7 Effect of Ca^{++} Concentration on Azyme Activity
Enzyme activity is plotted against Ca^{+} concentration

Requirement for reducing agent

Assays were done in the absence of DTT and in the presence of concentrations between 0.033 and 0.165 mM. DTT had little effect upon the activity. A small increase, about 10%, was observed at the highest DTT concentrations. These studies concern the assay conditions. Further studies would be needed regarding the role of DTT in stability during storage.

Stability studies

The partially purified enzyme was stored at 4^{o} at a series of pHs between 4 and 10. Neither DTT nor calcium was added. The enzyme decayed rapidly at pH 10. The pH 4 points were less active than the rest. There was little loss of activity even after 13 days of storage (Fig. 8). At 37^{o} the enzyme was less stable. The best stability was observed at pHs between 5 and 6 (Fig. 9). The reactions were in 0.02% sodium azide. The stability of affinity purified Azyme and crude material prepared by gel filtration was compared at 4^{o} and at 37^{o} at pH 6 in the absence of DTT and Ca^{++} (Fig. 10). The enzyme appeared fairly stable at 4^{o} but about half of the activity was lost in 12 hr at 37^{o}. It remains to be determined whether Ca^{++}, protein and/or DTT would stabilize the activity. Bovine serum albumin (0.1%) added to the assay of the affinity purified enzyme caused a 17% increase in activity suggesting that it might be useful in increasing the enzyme stability.

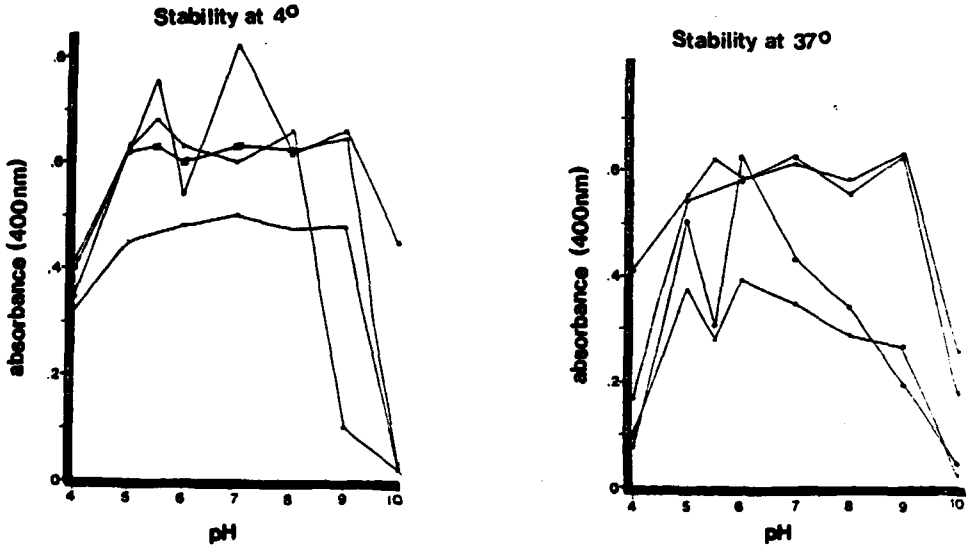


Fig. 8 Stability of Gel Filtered Azyme at 4^{o}
Azyme purified by gel filtration was stored for 0 hr (●), 47.5 hr (○), 143.5 hr (□) and 311 hr (■) at various pHs in the absence of added Ca^{++} and DTT. The absorbance is plotted against the pH.

Fig. 9 Stability of Gel Filtered Azyme at 37^{o}
Conditions as in Fig 8 except temperature and times, 0 hr (●), 2.3 hr (○), 47.5 hr (◘) and 144 hr (□).

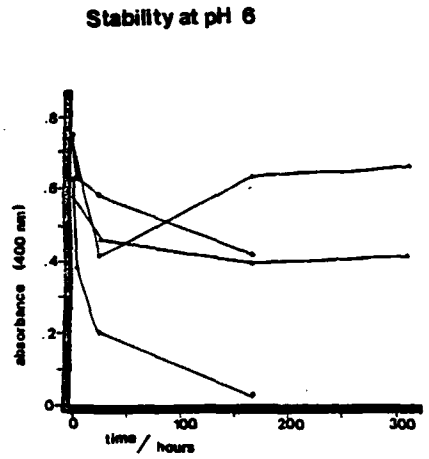


Fig. 10 Stability of Affinity Purified and Gel Purified Azyme

The reactivity of samples of clostridial enzymes after various times of storage. Squares represent data from gel filtered enzyme and circles that from the affinity purified material. Solid symbols are from 4° and open ones from 37°.

Determination of K_I for N-acetylgalactosamine

The apparent Km was determined at 5 N-acetylgalactosamine concentrations between 2 and 50 mM at 37° and the K_I calculated. The K_I was 1.38 mM. If one calculates the maximum amount of end product formed during a typical red cell conversion experiment, it is several orders of magnitude below the K_I. This suggests that end product inhibition plays no part in the slowing observed as the red cell conversion proceeds.

Effect of Hg^{++} on enzyme activity

As found by Paoni, Hg^{++} is a very effective inhibitor of the clostridial Azyme (Fig. 11).

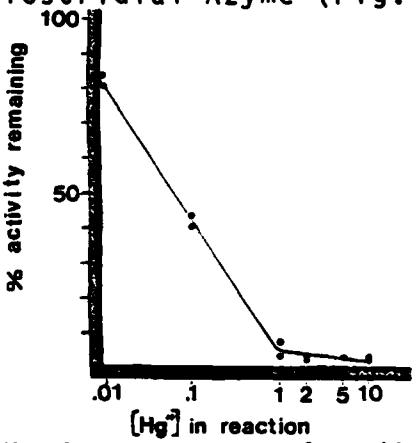


Fig. 11 Effect of Hg++ on Clostridial Azyme Activity

IV. Improvement of radioimmunoassay for A cells

Previous experiments had some difficulty with a high background count from the type O cells used as controls. This has been greatly decreased. In the previous progress report the background averaged 3.8% (range 1.4-9%). In the present experiments the background is consistantly less than 1%. Typical experiments had background counts of 43 for O cells compared to 9099 for A cells (0.47%) and 89/10123 (0.88%). The major reason for the improvement is an increase in wash volume. It is important to transfer the cells to a fresh container for counting as an appreciable number of counts are absorbed to the wall of the container (about 25% of input counts under typical conditions). The conditions of the

assay were also studied with respect to reaction volume and it was found independent of volume between 11 and 25 ul with a 13% decrease at 100 ul. Unlabeled monoclonal antibody displaced the labeled antibody. Five ul of unlabeled antibody displaced 3258 counts in a system which contained 6 ul of labeled antibody (7944 bound counts).

Of major importance is the demonstration of conversion. It validates the assay and makes it possible to readily compare the activities of the different Azymes on red cells compared to their activities on synthetic substrates. The value of a kinetic approach is demonstrated above where the leveling off of activity can be readily seen and the possible causes studied.

Plans for period from April 15, 1985 to April 14, 1986

The functional and validated assay for the conversion of type A red cells to type O allows one to follow the kinetics of conversion as well as its extent. A prime objective will be to determine why the conversion is not complete. As indicated we have ruled out enzyme inactivation and pH changes. Ineffective or incomplete digestion of one or more of the subtypes of A substance or one of the forms (protein or glycolipid) is another possibility. Small amounts of the subtypes of A substance are on hand and could be used for monoclonal antibody production so that the levels of Aa,Ab, Ac and Ad could be monitored during a digestion. A second possible cause would be the shielding of some residues ie. steric inaccessibility.

The assay will also give us an opportunity to optimize conditions for the conversion: temperature, time, rate of mixing, pH, RBC concentration. We will also determine whether Ca^{++} and DTT are needed for the clostridial enzyme. In addition to comparing the efficicy of the placental, liver and clostridial enzymes we will look at the effect of mixtures or sequential additions of different enzymes. It will allow us to compare the rates of red cell conversion with those for synthetic substrates ie. to determine the rate with the two substrates for each of the enzymes.

It is important to examine the conversion of A to O cells in as many ways as possible and preparations of the affinity purified enzymes will be sent to Dr. Jack Goldstein for studies in his laboratory.

The purification of the enzymes will proceed. In all affinity chromatography will be used. Studies optimizing Azyme formation in cultures and substituting synthetic media will continue. Purification of the clostridial enzyme is presently underway using the material partially purified by gel filtration. For elution of the absorbed enzyme, changes in salt concentration and pH will be used first followed by use of galactose and N-acetylgalactosamine as specific eluants if required. The presence of other hydrolases will be monitored as the fractionation proceeds. Emphasis will be placed on methods which can be easily scaled up.

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